STUDIES ON THE PRECIPITIN REACTION IN PLANTS

II. PRELIMINARY REPORT ON THE NATURE OF THE "NORMAL PRECIPITIN REACTION"

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IN 1928 Kostoff first called attention to the fact that the aqueous extracts of the foliage of certain Solanaceous plants precipitate in the presence of certain other such Solanaceous extracts (3). This precipitating action was designated as a "normal precipitin reaction," and the modification of such reactions after intergrafting underlies in part Kostoff's theory regarding acquired antibody production in such plants. In 1931 the writer published a record of his tests of the "normal precipitin reactions" in a number of species of the Oleaceae (1). These tests showed that the species of Oleaceae tested were in no case interreactive with the exception of numerous varieties of *Syringa vulgaris* which were suffering from a physiological blight due to graft incompatibility. Such varieties reacted strongly against a number of other Oleaceae including healthy, ungrafted plants of the same varieties as the reactive plants. Meanwhile Silberschmidt in Munich had also been studying the Kostoff phenomenon from the standpoint of technique. Silberschmidt has published an extended piece of research dealing mainly with improvements of the methods of extracting and testing (4), and he intended, at the time of publication, a continuation of his work dealing with the actual experimental results of his tests. Early in 1932 there appeared a second paper by the writer (2) showing that the "normal precipitin reactions" between species of the Rosaceae, Saxifragaceae, and Caprifoliaceae are well correlated with the systematic positions of the species considered.

Thus far little attempt had been made to determine the biochemical nature of the reaction. Kostoff and Silberschmidt both assume that the phenomena are of protein nature, and that they are analogous to the phenomena of animal serology. In 1932, however, the writer stated that: "Some of the reactions, such as those of *Prunus*, *Ribes*, and *Robinia*, lead one to the suspicion that possibly some non-specific compound is acting in a rather complex fashion to produce the precipitates" (2, page 71). The problem of the chemical nature of the "precipitin reaction" thus lay open to investigation and was of utmost importance in interpreting and evaluating the results of the earlier experiments. In the phytopathological laboratories of the Arnold Arboretum investigations have accordingly been in progress during the past winter to determine the chemical nature of the precipitating property of the extracts. As these investigations have been fruitful and as the results are of importance in directing future activities in this field, the present paper is designed to give a condensed account of these results, while a more detailed description of the work will appear in an early number of this Journal.

In the fall of 1931 collections of leaves of certain species of woody plants were dried and pulverized. These relatively homogeneous stock supplies were used for all the tests of woody plants described below. The technique employed was in all important particulars the same as that previously described (2). Among the species selected were *Prunus Armeniaca* var. "Mikado," *Platanus acerifolia*, *Robinia fertilis*, and × *Ribes Carrierei*. Of these four the *Prunus* tests strongly against the other three which latter are mutually inter-negative. The results obtained from a study of these four species were later found to apply to extracts of *Hydrangea paniculata* var. *grandiflora*, *Syringa vulgaris*, *Ligustrum obtusifolium*, *L. ibota*, and *L. vulgare* var. *foliosum*. Finally, the findings were applied to thirty-five species of Solanaceae, and accordingly the results here recounted have been gained from a study of practically all of the possible inter-reactions of forty-four species of herbaceous and woody plants.

As a working hypcthesis the theory was first entertained that the reactions might be of protein nature. Accordingly the earlier part of the investigation dealt with an analysis of the reactions on the basis of such an hypothesis. However, the results obtained from an analysis of the *Prunus-Robinia-Platanus-Ribes* reactions showed a very peculiar behavior if such an hypothesis were correct. The findings in this connection are here enumerated:

1. Variation in the salt content of the extracts by the use of Cohn's phosphate buffers at constant pH of 6.0 had very little effect on the reactive potency of *Prunus* between the limits of .06 M and 1.2 M. Between the same limits, however, there was a gradual fall in reactive potency of *Platanus* and *Robinia* from the weaker to the stronger salt concentrations.
2. Variation of the pH of the extracts by the use of Cohn's phosphate buffers at constant salt concentration of .06 M had no effect on *Robinia* between the limits of 5.2 and 8.4, and also no effect on *Prunus* between the same limits except for a sharp decline from 8.0 to 8.4 and from 5.6 to 5.2.
3. Long continued heating (even to three hours autoclaving at 5 lbs. pressure) did not decrease the reactive strength of either *Prunus* or *Robinia* extracts.

4. Precipitation of the extracts of *Prunus* or *Robinia* by excessive alkalinity or acidity, followed by filtration and subsequent neutralization did not remove the reactive principles.
5. There was no significant effect on the strength of the reaction if the pulverized leaves were thoroughly extracted with strong alcohol, anhydrous ether, benzol, or carbon tetrachloride as a preliminary to extraction with water.
6. There was no diminution in reactivity after storing the extracts at 2° C. for as much as four months. Even bacterial or fungous contamination had little or no effect on the strength or specificity of the reaction, when the extracts were subsequently cleared.
7. The progressive dilution of each of the extracts in turn showed an almost linear diminution in reactivity, the reaction disappearing in *Platanus* between dilutions of 1 : 256 and 1 : 512 and in *Prunus* between dilutions of 1 : 64 and 1 : 128. (*Note*: Normality of the extracts was arbitrarily chosen at 1 part dried tissue to 10 parts distilled water. The dilutions referred to here were further dilutions of such normal extracts.)
8. The reactivity of the extracts was unaffected by continued digestion with trypsin, pepsin, and yeast enzymes at appropriate pH values and temperatures.
9. Complete precipitation of *Prunus* by *Robinia*, *Platanus*, *Ribes*, or *Hydrangea* completely eliminated any further reaction of the thus-precipitated *Prunus* by any other of the latter four.
10. Fractionation of the extracts by the Rimington technique for removing carbohydrates showed that the reactive principle of *Prunus* was precipitated by neutral lead acetate and was entirely recoverable from that precipitate on treatment with H_2S. The reactive principles in *Robinia* and *Platanus*, on the other hand, were unaffected by treatment with both neutral and alkaline lead acetate and were completely recoverable in the nearly Molisch-negative filtrate from such treatment.
11. Extensive dialysis experiments showed that the reactive principles of *Prunus*, *Platanus*, and *Robinia* passed freely through membranes impermeable to formed proteins and only very slightly permeable to protein cleavage products. The strength of the reactive principles thus fractionated varied directly with the strength of chloride and carbohydrate (used as indices of the degree of dialysis) and bore no relation whatever to the strength of protein as indicated by the Millon and xanthoproteic tests (used as indices of the degree of dialysis).

Reviewing the evidence presented, the probability that the *Prunus-Platanus-Robinia-Ribes* reactions are due to proteins is very slight. Although plant proteins are more resistant to heat than animal proteins and accordingly the results in heating the extracts are inconclusive by themselves, yet it is inconceivable that proteins would not be removed or inactivated by treatment with acids and alkalis and with alcohol, by salt content and pH, by contaminations and enzyme action, and by the removal of various constituents of the extracts as must be the case if proteins are here involved. The identity of the various reactions of these species (item 9 above) argues against the protein hypothesis, and finally the experiments in dialysis show that it is extremely unlikely that the reactions are due to extremely small traces of protein. The likelihood that these reactions are due to lipoids or to carbohydrates is also very dubious in view of the results given in items 5 and 10 respectively.

In the course of an experiment on hydrolysis of the extracts a small excess of $CaCO_3$ was added to a sulphuric acid solution of *Prunus*. On neutralization it was found that its precipitating action was completely reversed. The presence of the calcium sulphate thus formed, although only very slightly soluble in water, rendered the *Prunus* negative to *Platanus*, *Robinia*, and *Ribes*, and, as was later found out, positive to extracts with which it had formerly been negative. Attention was accordingly directed to the inorganic constituents of these extracts with particular reference to calcium compounds.

This last was a most productive field. It was soon discovered and confirmed that the reactions in the woody plants under consideration are due to the interaction of free calcium ion in certain extracts (in this case in *Robinia*, *Platanus*, and *Ribes*) with free oxalate ion in the other extracts (represented for the present by *Prunus*). Such a view has resulted both from chemical analyses of the precipitates and from studies of the behavior of the whole extracts with regard to presence or absence of calcium and oxalate. The analytical evidence supporting the view that these reactions are due to the interaction of such ions in first presented:

1. The precipites, after washing in several changes of water, are white, heavy, limey, easily centrifuged, and inorganic in appearance. There is no charring on heating to 500° C.
2. The precipitates microscopically are in the form of regular granules, not amorphous, identical in appearance with certain commercial samples of calcium oxalate.
3. Recrystallization of the precipitates (by solution in strong H_2SO_4 and precipitation by neutralization with strong KOH) gives crystals of the characteristic size and shape of CaC_2O_4

crystals, and indistinguishable from crystals of a commercial sample of CaC_2O_4 similarly treated.

4. Treatment of the granules of the precipitates with strong H_2SO_4 under the microscope shows first a moderate solution followed by a very striking conversion of the remainder of the granules into the characteristic raphides of $CaSO_4$. This is a fairly accurate test for CaC_2O_4 and is precisely the behavior of a sample of commercial CaC_2O_4 similarly treated.
5. The precipitates are insoluble in all ordinary solvents. They are moderately soluble in strong H_2SO_2 but not in weaker acids. Their solubilities are thus equivalent to those of CaC_2O_4.
6. If alcohol is added to the H_2SO_4 solution of the precipitates there is a precipitation. (Test for calcium ion.)
7. The acid solution reduces potassium permanganate. (Test for oxalates).
8. Ignition of the washed precipitate yields 33% of oxide. The theoretical yield for CaC_2O_4 is 38%.

The evidence thus given leaves no room for doubt that the precipitates resulting from the addition of the *Prunus* extract to those of *Platanus*, *Robinia*, and *Ribes* consist of calcium oxalate. This evidence is still further confirmed by experiments to be reported below. At this time, however, certain questions arise: (a) Is this calcium oxalate reaction the only one involved in the combinations of woody plants under consideration? (b) How generally is the calcium oxalate reaction distributed through plants with especial reference to the tests which have been reported in the literature? (c) Are other reactions also involved in the tests which have been reported, and if so, what is their nature? The answers to these questions will appear from the following considerations.

If the calcium oxalate reaction is the main or only reaction in the woody plants available for this study it should be possible to divide all the extracts into two groups, a calcium-positive, oxalate-negative group which is intranegative but positive to a second, oxalate-positive, calcium-negative group. Such is possible. *Prunus Armeniaca* "Mikado" is here the only representative of the "oxalate" group (containing oxalate, lacking calcium), while the "calcium" group comprises *Platanus*, *Ribes*, *Robinia*, *Syringa*, *Hydrangea*, and the three species of *Ligustrum*. With the exception of a very weak reaction to be mentioned later, the latter group is perfectly intranegative. On the other hand, the members of this "calcium" group all produce precipitates identical in appearance with the "precipitin reaction" when they are added to weak solutions of oxalates ($K_2C_2O_4$ .01 M; $(NH_4)_2C_2O_4$ .02 M) but are inactive to the addition of weak solutions of calcium salts ($CaCl_2$ .005 M; $Ca(NO_3)_2$ .005 M). *Prunus*, however, reacts positively to pure solutions of calcium salts of the concentrations given above but is negative to these oxalates.

At this juncture it was felt advisable to extend these results to the Solanaceae for the dual purpose of obtaining more extensive data, and of obtaining data on the family on which the work of Kostoff and Silberschmidt has been done. Accordingly, all the possible interreactions of thirty-five species of Solanaceae as well as all their reactions with the woody plants under consideration were carried out by the writer in collaboration with Dr. Thomas Whitaker. The results of these tests were highly confirmatory. Testing the various Solanaceae against weak solutions of two oxalates and of two calcium salts it was found that the species fell into three groups, a group (Ca- Ox+) comprising *Atropa belladonna*, *Solanum tuberosum*, *Capsicum frutescens*, *Physalis peruviana*, *Salpiglossis sinuata*, *Datura ferox*, *D. metel*, and *D. innoxia* which were all positive in varying degree to the calcium salts and negative to the oxalates, a group (Ca- Ox-) comprising *Datura Wrightii*, *Cyphomandra betacea* and *Browallia viscosa* which were negative to both ions, and a group (Ca+ Ox-) comprising eighteen species of *Nicotiana*, *Petunia violacea*, *Lycopersicum cerasiforme*, *Solanum capsicastrum*, *S. melongena*, and *S. nigrum* which were negative to free calcium ion but positive to free oxalate ion. The extracts of group (Ca- Ox+) were positive to those in group (Ca+ Ox-) but those of group (Ca- Ox-) were negative to all in both other groups. Moreover the strength of the "precipitin reactions" bore a very close relation to the strength of the reactions of the corresponding extracts with the pure salt solutions. The (Ca- Ox+) group was perfectly negative *inter se* and the (Ca+ Ox-) group was also negative *inter se* except for a few weak reactions principally involving *Datura Wrightii* and *Nicotiana Rusbyi*. Thus a consideration of the Solanaceae affords very strong confirmatory evidence as to the validity of the calcium oxalate explanation.

A warning should be introduced at this point. The writer is well aware of the fluctuations of the salt content in plant juices and makes no contention that the values for the reactions are definite and always exactly reproducible. The readings will vary with the observer, with the technique, and with the environment and heredity of the plants investigated. However, that high developments of the content of such inorganic constituents are characteristic of certain species is evident from a consideration of the literature. Moreover that the results here described are comparable is evident from the facts that the layerings and readings were all made by the same observer, that all the reactions described for any given species of plant in this study were made from one given extract, that the Solanaceae were all grown under uniform greenhouse conditions, and that all of the "precipitin" tests described were made within a few days of one other.

An important confirmation should now result from testing the "calcium" Solanaceous extracts against the woody "calcium" extracts. If the calcium oxalate explanation be correct and complete, then such reactions should all be negative. In order to extend the limits of this experiment still farther, all the "oxalate" extracts, woody and herbaceous, were treated with a slight excess of $CaCl_2$ and filtered. Hence the calcium oxalate reaction was completely eliminated from consideration. Then all the possible combinations of the 42 extracts were again tested. This experiment brought out a very important fact. There is a second reaction in which *Platanus*, *Robinia*, and *Ribes* show a varying degree of reactivity against all the Solanaceae. The reactions of *Platanus*, *Robinia*, and *Ribes* are so well correlated here that it is most probable that there is a single substance (A) present in these three extracts which reacts with a second substance (B) in the Solanaceae. *Prunus*, *Syringa*, *Ligustrum obtusifolium*, and *L. ibota* react against neither the (A+ B-) nor the (A- B+) groups and accordingly are assumed to contain neither reactive principle.

It is now possible to eliminate both the calcium oxalate and AB reactions from consideration by considering only the interreactions of the 39 oxalate-negative, A-negative extracts. If this group of interreactions be studied it is seen that the great majority are negative (87%). There are a few scattered weak reactions, however, principally involving *Nicotiana Rusbyi* and *Datura Wrightii*. Rearranging all the remaining extracts according to their strength of reaction against the similar *N. Rusbyi* and *D. Wrightii* we find again that the 39 extracts are divisible into three groups, a group (M+ N-) comprising *N. Rusbyi*, *D. Wrightii*, *Cyphomandra*, *Ligustrum ibota* (?), and *Prunus* (?) which are positive to a group (M- N+) containing the remainder of the species with the exception of *Browallia*, *Salpiglossis*, *Petunia*, *Syringa*, *Ligustrum vulgare*, and *Solanum nigrum* (M- N-) which are negative to both groups.

There still remain a very few weak reactions (2%) which may be interpreted as due to a substance (X) present in *Solanum capsicastrum*, *Atropa*, *Physalis*, *Datura innoxia*, and *D. metel* which reacts with a substance (Y) present in the Oleaceae studied, in *Browallia*, and in *Nicotiana suaveolens*, but absent in the other remaining extracts

These four reactions, the first proven, the second and third assuredly present and distinct but of unknown nature, and the fourth more problematical, since it is very weak, explain all of the thousand or fifteen hundred reactions considered in this study. Of all the positive reactions at least 57% are due to the calcium oxalate combination, approximately 23% to the AB reaction, 17% to the MN reaction, and less than 3% to the XY reaction.

We are now in a position to return to the questions propounded above. First, with regard to the presence of any other reaction in the *Prunus-Platanus-Robinia-Ribes* complex, we may say definitely that there is no other "precipitin" reaction demonstrable by the technique employed than the calcium oxalate reaction. Removal of the calcium oxalate reaction leaves these four extracts perfectly internegative. Second, as to the distribution of the calcium oxalate reaction among the experimental plants heretofore studied it may be said with certainty that this reaction is of wide distribution, that it accounts for the majority of the reactions of the plants used by Kostoff, and that it must be eliminated before any immunological interpretation can be made of the data thus far published. Kostoff published a table of "normal precipitin reactions" in the Solanaceae which includes 56 positive reactions. 40 of these have been repeated in this laboratory. Of the 40 repetitions, in 7 cases there were no positive results, in 10 cases the reactions were due to the MN combination, and in 23 cases the reactions were due to calcium oxalate. Kostoff reported acquirement of "precipitin potency" in 12 instances. The normal reactions as repeated in this study show that of the 8 repetitions 3 reactions were due to calcium oxalate, 3 were due to the MN reaction, and in 2 cases no positive results were obtained. Kostoff found a decrease of "precipitin potency" in 4 cases. 3 of these have been repeated and in all 3 cases the normal reactions were found to be due to the MN combination.

Third, as regards the presence and nature of other reactions in the plants considered, it may be said with certainty that there is at least one other reaction present, probably two, and possibly three or more. The nature of these other reactions is being investigated. For the time being, it may merely be said that with regard to the AB and MN reactions they are indubitably organic in nature since the washed precipitates are strongly charred on ignition. The precipitates of these reactions are very different in appearance from the calcium oxalate precipitate. They are brown in color, even after washing, are copious and flocculent, and easily pass into a non-filterable colloidal suspension in pure water.

As further proof of the presence of more than one reaction, instances may be mentioned in which given extracts may be precipitated thoroughly to remove the calcium oxalate factor. Such calcium oxalate free extracts have lost none of their potency for precipitating in the presence of the opposite principle of the AB or MN reactions.

A word should be inserted at this point regarding the expressions "calcium" extracts, "oxalate" extracts, etc., frequently used in the foregoing pages. Such expressions do not imply that certain *plants* are free from demonstrable oxalate or calcium respectively, since the extracts used reveal the *excess* of either ion after such autoprecipitation of CaC_2O_4 as may take place in preparing the extracts. It is very apparent, for example, that *Prunus Armeniaca* vars. "Mikado" and *ansu* fall into the "oxalate" class not because they lack calcium but because they contain more than sufficient oxalate to neutralize the calcium normally present in the leaves.

With regard to the interpretation of the writer's earlier experiments in the light of the present findings, two questions arise: (a) Is the reaction of graft-blighted Lilac toward healthy Oleaceae (1) susceptible to explanation according to the calcium oxalate interpretation? (b) What light do the present findings throw on the specificity of the reactions as found in the Rosaceae et al. (2)? Both of these questions are readily understood by the results of the present paper.

In the first place, normal Lilac, and indeed the other species of Oleaceae studied, are all "calcium" plants, i. e. their extracts contain an excess of Ca^{++} ion. During the actual dying of the leaves of blighted Lilac there is an accumulation of oxalic acid or an oxalate in the affected cells. That this is not generally distributed throughout the plant (as would be true of an immunological substance resulting from grafting) is shown by the fact that the extracts made from green areas of mottled green-and-yellow leaves (blighted) test just as do normal leaves of ungrafted plants. The accumulation of free oxalate in the blighted portions of the leaves is in harmony with the fact that such leaves when ground and mixed with water begin an autoprecipitation (of the calcium normally present in the leaf by the oxalate) which if permitted to be carried to the end results in an excess of unused oxalate which gives the reaction with the other Oleaceae previously described. Immediately after maceration of the leaves, however, both oxalate and calcium can be demonstrated in the extracts.

Regarding the second question, as to the specificity of the reactions thus far observed, light was thrown upon this phase of the work by a consideration of the distribution of free calcium ion, free oxalate ion, and the other reactive principles in these various species of plants studied. On purely *a priori* grounds it would not be unreasonable to expect that in general the distribution of such substances would show some agreement with the taxonomic relationships of the plants involved. Thus it is well known that the presence or absence of calcium oxalate crystals in plant cells is of taxonomic significance. How does this apply to the Solanaceae and woody plants here studied? A dendritic chart was made including all the possible combinations of reactive substances believed to be responsible for the reactions observed. Thus the chart would first include a trifurcation representing presence of free calcium or free oxalate or absence of both. Each subdivision could then be further trifurcated according to presence or absence of the A and B principles, etc. There would thus be 81 possible combinations of reactive principles represented. If the assumption of specificity be correct, there should be a definite tendency for related species to have related positions on such a chart. The actual placement of the various species in their proper positions on such a chart brought out the validity of this assumption in striking clearness. 18 species of *Nicotiana* had been studied. All contain free calcium but not free oxalate, all contain the principle B but lack the principle A, *N. Rusbyi* alone contains the principle M, the other 17 species all contain N, while all the species of *Nicotiana* save *N. suaveolens* lack both X and Y. The possibility of their being so constituted by chance is infinitesimal. Similarly the Oleaceae considered are closely allied in all containing Ca^{++}, all lacking principles A and B except *L. vulgare* which has only a trace of this, all lacking both X and Y, and separating only with regard to the presence or absence of M and N. *Robinia*, *Platanus*, and *Ribes*, in closely related families, all are found to lie in the same quarter of the dendritic system, being all positive for calcium and for principle A, they being the only representatives of the A principle in the whole scheme. So, too, the 4 species of *Datura* as well as those of *Solanum* are found to agree in three of the four reactive principles, separating on only one.

It is thus seen that the distribution of the reactive principles in the 42 species of plants here considered is in good accordance with the taxonomic positions of these species, which clearly explains the earlier findings, before the nature of the reactions had been investigated, with regard to the specificity exhibited by the reaction.

SUMMARY

1. The present paper gives a condensed account of the writer's investigations of the biochemical nature of the "normal precipitin reaction" in plants.
2. Extensive experiments in the testing of various physical and chemical treatments have yielded strong evidence against the hypothesis that the reaction in certain woody plants, viz. *Prunus Armeniaca*, *Platanus acerifolia*, *Robinia fertilis*, and *Ribes Carrierei*, is of protein nature.
3. Conclusive proof is given that the reaction resulting in approximately 57% of the precipitates observed in all the possible combinations of 42 species of Solanaceous and woody plants is the precipitation of calcium oxalate by the interaction of the respective ions in pairs of extracts. This precipitation of calcium oxalate is so frequently the sole or main phenomenon in the reactions hitherto considered immunological in nature as to invalidate immunological interpretations laid on such reactions in which the calcium oxalate factor is not eliminated.
4. The remaining reactions are susceptible to interpretation on the assumption of the presence or absence of three other pairs of reactive substances. The nature of these reactive pairs is relatively obscure at present, but it is being investigated. One such pair (AB) is particularly characterized by the reactions of oxalate-free *Robinia*, *Platanus*, and *Ribes* with the Solanaceae. This reaction accounts for about 23% of the positive precipitations. It is shown to be distinct from the calcium oxalate reaction. A second pair (MN) is represented by the reactions of oxalate-free and AB-free *Nicotiana Rusbyi* and *Datura Wrightii* with most of the other Solanaceae. It is plainly distinct from the calcium oxalate and AB reactions and accounts for about 17% of the precipitations. A very small residue of reactions (less than 3%) is finally explained by the assumption of a last pair of reactive substances XY, the reactions being represented by those of *Solanum capsicastrum*, *Atropa belladonna*, *Physalis peruviana*, *Datura innoxia*, and *D. metel* against certain Oleaceae.
5. The writer's earlier findings with regard to "precipitin reactions" of physiologically blighted Lilac and to the specificity of the "normal precipitin reaction" are readily interpreted on the basis of the findings of the present study.

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